STUDIES OF THE FUNCTIONS OF THE LYMPHOID CELLS OF THE CHICKEN

With special reference to the effects of bursectomy and thymectomy

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AKADEMISK AVHANDLING
som med vederbörligt tillstånd av
Kanslern för Rikets Universitet
för vinnande av medicine doktorsgrad
framlägges för offentlig granskning
i Anatomiska institutionens föreläsningssal
torsdagen den 28 maj 1970 kl. 9.15 f.m.

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This dissertation is a summary of the following communications:

I. Alm, G.V. and Peterson, R.D.A. Antibody and immunoglobulin production at the cellular level in bursectomized-irradiated chickens. J. Exp. Med. 129: 1247-1259, 1969.

II. Alm, G.V. *In vitro* studies of chicken lymphoid cells. I. Phytohemagglutinin induced DNA, RNA and protein synthesis in spleen cells from control-irradiated and bursectomized-irradiated chickens. Acta Path. Microbiol. Scand. In press (condensed).

III. Alm, G.V. The *in vivo* spleen response to sheep erythrocytes in bursectomized-irradiated chickens. Acta Path. Microbiol. Scand. In press (condensed).

IV. Alm, G.V. *In vitro* studies of chicken lymphoid cells. II. The anamnestic response of spleen cells from control-irradiated and bursectomized-irradiated chickens to sheep erythrocytes.

V. Alm, G.V. *In vitro* studies of chicken lymphoid cells. III. The mixed spleen leukocyte reaction with special reference to the effect of bursectomy.

VI. Alm, G.V. *In vitro* studies of chicken lymphoid cells. IV. The effect of thymectomy on the antibody response *in vivo* and on the *in vitro* reactivity of spleen cells to sheep erythrocytes, allogeneic cells and phytohemagglutinin.

These papers will be referred to by the Roman numerals I-VI in the text.

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INTRODUCTION

The avian and mammalian thymus and the avian bursa of Fabricius have long been recognized as sites of lymphopoiesis (1-6).

Direct evidences for a role of these central lymphoid organs in the ontogenetic development and maintenance of peripheral lymphoid tissue, e.g. the lymphocytes in the circulation, the lymph nodes and the spleen and in the development of normal immunologic responsiveness have more recently been forthcoming.

Thus Glick and collaborators (7,8) discovered that chickens surgically bursectomized in the newly hatched period were deficient antibody producers as they matured. These findings initiated the experimental definition of the immunologic role of the bursa of Fabricius, and the thymus.

It was found that neonatal removal of the thymus in rodents suppressed antibody formation and cellular immune reactions in later life (9-13).

Avian species, represented by the chicken, are unique in as much as they have both a thymus and the bursa of Fabricius. The latter structure does not exist in mammals as a clearly defined organ although some experimental evidence suggest that gut-associated lymphoid tissues such as the Peyers patches may be a bursa equivalent (14). The gut epithelium of mammals have also been proposed as a bursa equivalent (15).

Warner et al. (16) originally suggested that the avian thymus and bursa of Fabricius, though both central lymphoid tissues, have basically different influences on the ontogenesis of the immune mechanism of the chicken.

This concept, supported by further experimental evidence from several laboratories (see 17-19 for reviews), suggested the existence of thymus-dependent and bursa-dependent populations of lymphoid cells. The work of Cooper et al. (20,21) sharpened this delineation of the lymphoid system further.

The components of the bursa-dependent system are the bursa itself and the germinal centers and the plasma cells of peripheral lymphoid tissue and its functions are immunoglobulin and specific antibody production ("humoral immunity").

The components of the thymus-dependent system are the thymus and many of the small lymphocytes in the circulation, in the splenic white pulp and other lymphoid tissues. These are mainly involved in expressions of cellular immunity such as the graft-versus-host reaction, delayed hypersensitivity and the homograft rejection. The thymus-dependent system may also be involved in the antibody response because thymectomy has by some workers been reported to depress the antibody production (20). Much of the literature on the avian thymus and bursa of Fabricius and their roles in the ontogenesis of lymphoid tissue structure and function has been reviewed by Warner (17), Cooper et al. (18) and Jankovic (19).

The chicken may provide an excellent opportunity for the study of the properties and functions of lymphoid cells, in particular the thymus-dependent lymphoid cells, in the absence of immunoglobulin and specific antibody formation produced by bursectomy.

A series of investigations were initiated with the following main purposes:

1. To evaluate the immune response, particularly the antibody and immunoglobulin producing capacity at the cellular level in chickens surgically bursectomized and sublethally irradiated at hatch. This was necessary because to our knowledge no such studies had previously been undertaken and because of recent discouraging reports relative to the incomplete effects of bursectomy on the antibody response and serum immunoglobulin levels (22).

2. To develop techniques suitable for study in tissue culture of the reaction of lymphoid cells to the nonspecific lymphocyte mitogen phytohemagglutinin (PHA), to an antigen such as sheep erythrocytes (SRBC) and to allogeneic lymphoid cells.

3. To evaluate to what degree these *in vitro* reactions were a function of thymus- and bursa-dependent lymphoid cells.

RESULTS AND DISCUSSION

1. ANTIBODY AND IMMUNOGLOBULIN PRODUCTION AT THE CELLULAR LEVEL IN BURSECTOMIZED-IRRADIATED CHICKENS.

The purpose of this investigation was to define the immunologic deficiency of the bursectomized-irradiated chicken with special reference to antibody response and immunoglobulin production.

Newly hatched white leghorn chickens were surgically bursectomized and sublethally x-irradiated (600 or 650 R) mainly according to Cooper *et al.* (21). Control chickens received x-irradiation only. At 8 weeks of age plasma immunoglobulin levels were assayed semi-quantitatively with immunoelectrophoresis and immunodiffusion. The ability of spleen cells to synthesize H^3-leucine labelled immunoglobulin *in vitro* was assayed by a coprecipitation technique. The presence of immunoglobulin antigenic determinants on spleen cells was assayed by the technique of Sell and Gell (23) which is based on the stimulation of the H^3-thymidine incorporation into the DNA of lymphoid cells cultured in the presence of anti-immunoglobulin serum. The capacity to produce antibodies was determined at the serum level by assaying for agglutinins to Brucella abortus antigen. At the cellular level antibody synthesis to sheep erythrocytes (SRBC) was assayed by the technique of local hemolysis in gel according to Jerne *et al.* (44) using both the original direct version which detect highly efficient, mostly IgM, hemolysin producing cells and an indirect version which, as employed, theoretically detects antibodies of all immunoglobulin classes.

The response of spleen cells to the lymphocyte mitogen phytohemagglutinin (PHA) in tissue culture was determined by the H^3-thymidine incorporation into DNA.

A significant proportion of the bursectomized-irradiated (Bx) chickens (26/151) had no detectable or trace levels of serum immunoglobulins. Spleen cells from such

animals synthesized little coprecipitable immunoglobulin. These cells also contained less of the antigenic determinants of immunoglobulins, shown by their decreased stimulation in tissue culture by anti-immunoglobulin serum but they appeared to react normally to PHA.

Bx chickens showed no antibody response to Brucella abortus and their spleens contained no cells forming antibody to sheep erythrocytes detected by the direct or indirect Jerne technique.

The results indicate that bursectomy and sublethal x-irradiation indeed in a significant proportion of chickens prevents the development of the antibody and immunoglobulin producing cells but probably does not affect the PHA reactive cells. The latter may not be immunoglobulin producers.

The results agree with those independently presented by Ivanyi et al. (24) with respect to the lymphocyte transformation by anti-immunoglobulin serum and Meuwissen et al. (25) with respect to the PHA response.

II. IN VITRO STUDIES OF CHICKEN LYMPHOID CELLS. I. PHYTOHEMAGGLUTININ INDUCED DNA, RNA AND PROTEIN SYNTHESIS IN SPLEEN CELLS FROM CONTROL-IRRADIATED AND BURSECTOMIZED-IRRADIATED CHICKENS.

In paper I was described the apparent deficiency of the spleen lymphoid cells of the Bx chicken with respect to immunoglobulin and antibody synthesis. A clear indication was obtained that the ability of such cells to respond to the lymphocyte mitogen phytohemagglutinin (PHA) was intact. The action of PHA on lymphoid cells has recently been reviewed (26).

Because of the potential importance of this observation and the findings that humans with acquired agammaglobulinemia were deficient in the response to PHA (45) a more extensive investigation of the PHA response of spleen lymphoid cells from hypogammaglobulinemic bursectomized-irradiated chickens was undertaken.

A tissue culture technique suitable for the study of the dynamics of the PHA induced changes of cell metabolism was developed. The DNA, RNA and proteinsynthesis in cultured spleen cells was monitored during a 48 hour culture period by the incorporation of H^3-thymidine, H^3-uridine and H^3-leucine respectively.

The RNA and protein synthesis increased in an approximately linear fashion for at least 24 hours, beginning within 4 hours after the addition of PHA. The increase of the DNA synthesis started first after approximately 16 hours but reached rapidly a maximum at 36 hours.

The responses to PHA of the spleen cells from hypogammaglobulinemic bursectomized-irradiated (Bx) and control-irradiated chickens were similar. The only exception was the PHA induced rate of RNA synthesis, determined by 4 hour pulses of H^3-uridine, which was higher in the Bx animals.

On the basis of these results and those in paper I it is suggested that the PHA reactive lymphoid cells are belonging to another cell population than the bursa-dependent immunoglobulin and antibody producing cells. Similar conclusions were reached by Meuwissen et al. (25) and with a different approach by Daguillard and Richter (26).

III. THE IN VIVO SPLEEN RESPONSE TO SHEEP ERYTHROCYTES IN BURSECTOMIZED-IRRADIATED CHICKENS.

The spleen of the normal chicken shows a response to intravenous immunization with sheep erythrocytes (SRBC) characterized by increased spleen weight, spleen cell number and H^3-thymidine uptake as an indicator of cell proliferation. Most of this cannot be accounted for by actively antibody forming cells. Because in the mouse thymus-derived lymphoid cells have been shown to proliferate to antigen without producing antibody (28,29) the possibility was considered that the thymus-dependent cells in the chickens might contribute to the in vivo response of the spleen to antigen. Bursa-dependent cells could also conceivably proliferate, but not form antibody. To test this, SRBC (5×10^9/kg bodyweight) were injected i.v. into bursectomized-irradiated (Bx) and control-irradiated (Cx) chickens; using unimmunized animals as controls. After 4 days an i.v. pulse of H^3-thymidine was administered. Four hours later the isotope incorporation per 10^6 spleen cells and in the whole spleen, as well as the total spleen weight, spleen cell number and body weights were recorded. Antibody forming spleen cells were determined by the direct and indirect versions of the Jerne technique.

Significant increases of spleen weights, cell numbers, H^3-thymidine incorporation and antibody forming cells were found in the immunized Cx but not in the Bx animals.

A further finding was that the comparative low numbers of spleen cells forming antibody to SRBC found in unimmunized Cx chicken were not present in Bx chickens. These results further demonstrated the extent of the immune deficiency in the Bx chicken indicating the absence of cells reacting to antigen by proliferation as well as antibody formation. They further suggest that the thymus-dependent spleen cells either contribute little to the normal in vivo proliferative spleen cell response after antigen stimulus or so is their proliferative response also deficient in the Bx chicken.

IV. IN VITRO STUDIES OF CHICKEN LYMPHOID CELLS. II. THE ANAMNESTIC RESPONSE OF SPLEEN CELLS FROM CONTROL-IRRADIATED AND BURSECTOMIZED-IRRADIATED CHICKENS TO SHEEP ERYTHROCYTES.

The in vitro anamnestic (secondary) immune response of lymphoid cells to many different antigens has been studied in several species using different tissue culture techniques (30). It is characterized by the development of antibody forming cells and the proliferation of lymphoid cells, only a small fraction of which are antibody producers.

In this investigation a technique was developed suitable for the study of the in vitro anamnestic response of chicken spleen cells to sheep erythrocytes (SRBC). Chickens were immunized intravenously with SRBC and 8 days later suspensions of spleen cells were prepared and cultured in stationary closed glass tubes, with or without the antigen. In vitro cell proliferation was indicated by the incorporation of H^3-thymidine into DNA. Antibody forming cells were detected by the direct and indirect versions of the Jerne technique.

It was found that these parameters were markedly, but differentially, dependent on SRBC antigen concentration and culture time. Thus the development of antibody forming cells, in particular of the direct type, was inhibited by high antigen-concentrations which caused no decrease of the DNA synthesis. The DNA synthesis and the "direct" and "indirect" antibody forming cells in the cultures showed maxima at different times of culture.

Spleen cells from immunized hypogammaglobulinemic bursectomized-irradiated chickens showed no significant in vitro anamnestic immune response to SRBC.

The simplest explanation for this finding is that all proliferating lymphoid cells, including the antibody forming cells, normally seen in the in vitro response are bursa-dependent. If many of them are thymus-dependent as shown in the mouse for the anamnestic immune response in vivo (29), it is possible that functioning bursa-dependent cells are necessary for the in vivo development of sensitized thymus-dependent lymphoid cells or for the in vitro initiation of their proliferation. The latter could be achieved in a "nonspecific" way by antigen-antibody complexes (31,32).

V. IN VITRO STUDIES OF CHICKEN LYMPHOID CELLS. IV. THE MIXED SPLEEN LEUKOCYTE REACTION, WITH SPECIAL REFERENCE TO THE EFFECT OF BURSECTOMY.

The mixed leukocyte reaction (MLR) which occurs between two allogeneic lymphoid cell populations in vitro was first described by Bain et al. (33). The MLR is immunologic in nature and based on differences in the major histocompatibility antigens. It may be an expression of cellular immunity (34). Lymphocytes from patients with Bruton's type agammaglobulinemia were either normal (35) or deficient (36) in the MLR.

In the present investigation the hypogammaglobulinemic bursectomized-irradiated chicken was used on an experimental model to establish whether antibody forming bursa-dependent lymphoid cells play any role in the mixed leukocyte reaction (MLR).

The tissue culture technique was similar to that in paper IV. Two versions of the MLR was used. Equal numbers of untreated spleen cells from two allogeneic chickens were mixed (two-way MLR), or one of the two cell populations were pre-treated with mitomycin C (one-way MLR) according to Bach and Voynow (37). The degree of stimulation by allogeneic cells was measured by the incorporation of H^3-thymidine (4 hour pulse, on day 5 usually). The effects of the cell concentration in the cultures and of the culture time on the MLR were evaluated.

A modification of the indirect version of the Jerne technique, with chicken erythrocytes substituting for the sheep erythrocytes, was used in an attempt to detect cells forming antibody to histocompatibility antigens in the MLR.

Finally the reactivity of spleen cells from control-irradiated (Cx) and hypogammaglobulinemic bursectomized-irradiated (Bx) chickens in the one-way and two-way MLR was compared.

The results showed that the tissue culture system was suitable for the study of the MLR. No cells forming antibody to histocompatibility antigens were detected in the cultures. Spleen cells from Bx chickens responded and stimulated normally in the MLR.

Therefore the bursa-dependent lymphoid cells most likely do not play an important role in the mixed leukocyte reaction in vitro.

VI. IN VITRO STUDIES OF CHICKEN LYMPHOID CELLS. IV. THE EFFECT OF THYMECTOMY ON THE ANTIBODY RESPONSE IN VIVO AND ON THE IN VITRO REACTIVITY OF SPLEEN CELLS TO SHEEP ERYTHROCYTES, ALLOGENEIC CELLS AND PHYTOHEMAGGLUTININ.

The previous investigations (I-V) dealt with the functions of the lymphoid cells in the bursectomized-irradiated chicken. In this report (VI) the effect of thymectomy on the antibody response to sheep erythrocytes (SRBC) and on the in vitro proliferative response of spleen cells to SRBC, allogeneic spleen cells and PHA was evaluated.

Newly hatched chickens were surgically thymectomized and given 600 R x-irradiation. Sham-thymectomized animals, given the same x-ray dose, were the controls. At 8 weeks of age half of the animals received SRBC i.v. and all were sacrificed 8 days later. The serum titers of hemagglutinins to SRBC were determined. Spleen antibody forming cells were assayed by the direct and indirect versions of the Jerne technique. The responses of spleen cells to PHA, SRBC and allogeneic spleen cells (one-way MLR) were studied with the tissue culture techniques described in papers IV and V.

Unimmunized thymectomized-irradiated (Tx) chickens showed significantly elevated serum hemagglutinin titers and increased numbers of "direct" antibody forming cells in the spleens compared to unimmunized sham-thymectomized-irradiated (Cx) chickens. Immunization with SRBC increased the hemagglutinin titers and the number of "direct"

and "indirect" antibody forming cells per spleen only in the Cx, but not the Tx groups. However, no direct difference could be demonstrated in the titers of the antibody forming cell numbers between immunized Cx and Tx animals.

The spleen cell response *in vitro* to PHA and to allogeneic spleen cells in the one-way MLR was significantly impaired in Tx chickens, the former possibly more than the latter. The SRBC immunized Cx, but not the Tx, chickens showed a significant proliferative response to SRBC *in vitro* compared to unimmunized controls. A direct difference between the magnitude of the responses to SRBC of the immunized Cx and Tx chickens could not be convincingly demonstrated.

The cause of the increased serum SRBC hemagglutinins and the number of spleen cells forming antibody to SRBC in unimmunized Tx chickens is unknown. One possibility is infection by organisms, particularly bacteria, that immunologically cross-react with SRBC (38).

The failure of deliberate immunization with SRBC to increase the serum hemagglutinin titers and the number of antibody forming cells in the spleens in Tx chickens can be explained in several ways. One possibility is that the thymus-dependent cells in the chicken, as the thymus-derived cells in the mouse (39,40,41), play a role in the induction of the antibody response. The possibilities of an inhibitory effect of preexisting antibody (42) or competition with (unidentified) antigens (43) cannot be excluded.

Because thymectomy combined with x-irradiation inhibited the responses to PHA and allogeneic lymphoid cells, these may be functions of thymus-dependent cells. The effect of thymectomy on the proliferative response to SRBC may indicate that thymus-dependent cells are involved in the *in vivo* development of immune cells or/and directly in the *in vitro* response to the antigen.

GENERAL SUMMARY AND CONCLUSIONS

In most, the results of this series of investigations support the concept of two components of lymphoid tissue as outlined in the introduction. They demonstrate the completeness with which the development of the lymphoid tissue and its functions are prevented by early surgical bursectomy or thymectomy combined with sublethal x-irradiation.

It would appear that the functions of the bursa-dependent lymphoid cells is the synthesis of immunoglobulin and specific antibody. They respond poorly or not at all *in vitro* to phytohemagglutinin and allogeneic lymphoid cells. The thymus-dependent lymphoid cells in contrast synthesize little or no immunoglobulin or antibody but respond well to phytohemagglutinin and allogeneic lymphoid cells.

Both the *in vivo* primary immune response and the *in vitro* anamnestic (secondary)

response of spleen cells to sheep erythrocytes, measured as cell proliferation and antibody formation was absent in the bursectomized-irradiated chickens. This suggested that the thymus-dependent cells were not stimulated to a significant degree under the experimental conditions. The finding that also thymectomized-irradiated chickens, although less impressive, showed deficient *in vivo* antibody response and *in vitro* proliferative responses to sheep erythrocytes suggest that perhaps the bursa and thymus-dependent systems of lymphoid cells in some way interact in the primary and/or secondary immune responses to this antigen.

ACKNOWLEDGMENTS

These investigations were carried out at the La Rabida-University of Chicago Institute, University of Chicago, Chicago, Illinois, U.S.A. I thank Professor Raymond D. A. Peterson, M.D., who introduced me to the subject while at the Department of Histology in Uppsala, and later placed his laboratory facilities in Chicago at my disposal. His kind interest and advice greatly contributed to my work.

I am indebted to Professor K. E. Fichtelius, M.D., Department of Histology, Univer of Uppsala, who introduced me to lymphocyte research, for advice and encouragement during these investigations.

I am most grateful to Professor Bo Hellman, M.D., Department of Histology, Univers of Umeå, Professor Sven E. Brolin, M.D. and Associate Professor Claes Hellerström, M.D., Department of Histology. University of Uppsala, who guided my first steps into research.

I gratefully acknowledge the advice of Assistant Professor L. W. Coppleson, M.D., Department of Surgery in statistics.

Finally, I wish to thank faculty and staff of the Department of Histology, Univers of Uppsala and the La Rabida-University of Chicago Institute who in many ways facilitated my work. In particular, I wish to acknowledge the excellent and devoted technical assistance of Mrs. Clara Maynie.

The investigations have been supported by Svenska Sällskapet för Medicinsk Forsknin the Medical Faculty, University of Uppsala, Petrus och Augusta Hedlunds stiftelse and the United States Public Health Service (AI-08771-01 and 5-T01-AM-05589-2).

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